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OPTIMIZATION OF GROWTH AND TOLUENE DEGRADATION BY FREE AND IMMOBILIZED *BACILLUS CEREUS* ATHH39

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Abstract

A toluene-degrading bacterium with high tolerance of toluene was isolated from oil-contaminated soils. DNA sequencing and homologous analysis identified it as *Bacillus cereus* sp. Toluene degradation was optimized in respect to pH, temperature and toluene concentration using response surface methodology with central composite design. At optimal pH (6.7), temperature (33°C) and toluene concentration (825 mg/L) predicted degradation was 65.5%. Carbon nanotubes were used to immobilize the *Bacillus*; immobilized cells degraded toluene by 87.5% tolerated a higher toluene levels and protected the bacteria against changes in temperature and pH. These results indicate that immobilized *Bacillus cereus* strain ATHH39 possesses a good application potential in the treatment of toluene-containing soils.

Introduction

Monoaromatic hydrocarbons (BTEX) are a group of hazardous pollutants which originate from sources such as drilling, storage, transport, refineries, gas, and oil extraction fields, petrochemicals and paint and glue industries (SEIFI et al. 2011). This oily waste poses a crucial environmen-

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tal pollutant and creates the problem of ground water contamination due to improper disposal/discharge on nearby lands of refineries (HU et al. 2013). They exert harmful and toxic effect on the environment and have been classified as hazardous chemicals due to carcinogenic and mutagenic nature. Degradation of such compounds is on top priority work, needs safer, and environmentally friendly treatment method. During the last decade, much research has been carried out on treatment technology for oily sludge management, which includes land farming, incineration, solidification/stabilization, solvent extraction, ultrasonic treatment, pyrolysis, photocatalysis, chemical treatment, and biodegradation (HU et al. 2013). But no single method is capable of removing/degrading total components of oily sludge. One of the most effective of these methods is adsorption because processes based on this concept are simple, highly efficient, and easy to operate; therefore, adsorption processes are widely used (JIA et al. 2013, LIU et al. 2013). Various adsorbents have been developed for the removal of organic pollutants from water (ATUL et al. 2013). Recently, a great deal of attention has been focused on the application of nano-structured materials as adsorbents to remove toxic and harmful organic substances from wastewater (ADITYA et al. 2011, MOHMOOD et al. 2013). Carbon nanotubes (CNTs), are one of the most widely studied carbon nanomaterials and can serve as excellent adsorbents (LATORRE et al. 2012) because of their hollow and layered structure and large specific surface area, which is why CNTs are the most commonly used nano-materials for adsorbing toxic material (SUI et al. 2012b, SWEETMAN et al. 2012). The use of immobilized cells or microorganisms in reactors offers many advantages over suspended cell systems, such as easier separation, greater operational flexibility, and higher cell density, resulting in higher rates of biodegradation per reactor unit volume (PARAMESWARAPPA et al. 2008). It has also been reported that immobilized cells are protected from harsh environmental conditions, becoming more tolerant to high concentrations of toxic compounds (CHEN et al. 2007). In this sense, there is a continuous search for more efficient, easier to handle, and lower cost supports. However, the degradation efficiency is decided by the environment, key factors like contaminant concentration, bioavailability, and catabolic strength of microflora, nutrient requirement, moisture level and geographical situations. Therefore, it is essential to study the simultaneous effects of different environmental conditions. Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for the modeling and problem analysis in which a response of interest is influenced by several variables and the objective is to optimize the response (MYERS et al. 2016). The central composite design (CCD) is the standard RSM and allows estimating the second-degree polynomial of the relationships between the independent variables and the dependent variable and gives information about the interaction between variables in relation to the dependent variable (ZHAO et al. 2017).

In this study, a bacterial strain that can degrade elevated concentration of toluene, *Bacillus cereus* strain ATHH39, was isolated and characterized. Moreover, multi-walled carbon nanotubes (MWCNTs) used to immobilize the strain ATHH39. Factors affecting a toluene degradation process and operational and store stability of immobilized cells investigated.

Materials and Methods

Medium and culture conditions

The toluene-degrading *Bacillus cereus* strain ATHH39 used in this study isolated by enrichment for growth on toluene as the sole carbon source from the oil contaminated soils of Caspian Sea (Bandar-Anzali Guilan, Iran). This strain was grown aerobically at 30°C mineral salt medium (MSM) containing (per liter) 4 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g Na_2HPO_4, 0.2 g $MgSO_4$, $7H_2O$, 0.0011 g $FeSO_4$, H_2O, 0.01 g $CaCl_2$ (Merck, Darmstadt, Germany) (ZHANG al. 2013). The medium was adjusted to pH 7.0 and autoclaved to sterilize at 121°C for 15 min. Bacterial isolate was grown 24 h at 30ºC, 150 rpm, in MSM broth supplemented with 1% (v/v) toluene (99.5% purity; Merck, Darmstadt, Germany) as the sole carbon and energy sources. Cells were harvested by centrifugation (10,000 × g for 10 min), washed twice in sterile MSM broth and resuspended in one-tenth volume of medium. Bacterial suspension with the density equal to 0.5 McFarland used as inoculum to predict the optimal medium composition for toluene degradation. The growth rate of the isolate was determined turbidometrically at 600 nm (MALATOVA 2005). The toluene degradation done by dissolving residual toluene of the medium in 3 ml n-hexane and reading the optical density against a blank at 200–400 nm wavelengths (WANG et al. 2008). All the experiments were carried out independently and the results were the average of three replicate experiments.

Identification of strain ATHH39 by 16S rDNA sequence

16S rDNA was amplified with the primers 27F-AGAGTTTGATCMTGGCTCAG and 1492R- GGTTACCTTGTTACGACTT. PCR reaction was done as originally described by MADUENO et al. (2011). 16S rDNA sequenced by the Iranian Biological Resource Center. The final sequence of 1500 bp submitted to the GeneBank under accession number KX344721. The sequence submitted to a BLAST search of the NCBI GeneBank database to identify the organism.

Optimization of the degradation process

Optimization of *Bacillus cereus* ATHH39 growth and toluene degradation carried out in MSM medium. RSM was employed to optimize pH (X_1), temperature [°C] (X_2), and toluene concentration [mg/L] (X_3). RSM with a three-factor, three-level CCD design used to optimize the response, Y (toluene degradation) of three variables:

$$Y=\beta_0+\sum_{i=1}^{3}\beta_i X_i+\sum_{i=1}^{3}\beta_{ii}X_i^2+\sum_{i=1}^{2}\sum_{j=1}^{3}\beta_{ij}X_iX_j \quad (1)$$

Where β_0, β_i, β_{ii} and β_{ij} are intercept, linear, quadratic and cross product regression terms, respectively. X_i and X_j are coded independent variables, linearly related to X_1, X_2, and X_3. The actual factor level corresponding to the coded factor levels are shown in Table 1. The ranges of factor levels for the experimental design selected based on the original medium.

Table 1

Levels and codes of variables for central composite design

Variables	Level code				
	-1.68	-1	0	1	+1.68
X_1	5.32	6	7	8	8.68
X_2	21.59	25	30	35	38.41
X_3	195.46	400	700	1000	1204.54

Explanation: X_1 – pH; X_2 – temperature [°C]; X_3 – toluene concentration [mg/L]

The optimal culture conditions for maximum toluene degradation estimated by statistical analysis using the Design Expert Software (version 7.1). The coefficients in the second-order polynomial (Eq. 1) calculated by multiple regression analysis on the experimentally obtained data.

Bacterial immobilization

MWCNTs with 5–10 nm inner and 20–30 nm outer diameter, surface area of > 110 m^2/g and purity above 98% were purchased from US Research Nanomaterials. The amount of 1 g of MWCNTs treated with 60 mL of a 3:1 mixture of nitric (15.6 mol/L) to sulfuric (14.8 M) acid and were dispersed using probe sonicator for 3 h (ZHANG et al. 2011, PETERSEN et al. 2010).

To prepare a stable stock solution, MWCNTs dispersed in deionized water by ultrasonication (200 W, Cole-Parmer CV33) for 30 min, and then the mixture left at room temperature. Then the solution was filtered through a 0.45 μm membrane filter and then washed with deionized water until the neutral pH. The treated MWCNTs dried for 12 h at 60°C and stored for further use (PAN et al. 2007).

10 μl of freshly adapted bacteria with the density equal to 0.5 McFarland were transferred in to 10-ml of MSM containing 100 μl of 0.05 g/L treated MWCNT and incubated at different cultural conditions. The quantities of toluene in liquid medium degraded by free and immobilized bacteria estimated by measuring the absorbance of the culture solution at 200–400 nm.

Free and Immobilized *Bacillus cereus* ATHH39 were analysed using Scanning Electron Microscopy (SEM). For SEM analysis, Free and Immobilized strain were rinsed three times with sterile distilled water.

Results and Discussion

Isolation and characterization of Bacillus cereus strain ATHH39

By enrichment culture, the strain ATHH39 was selected for detailed studies because of its high toluene-degrading rate and capable of growth on toluene as the sole carbon and energy source. Microorganisms, in addition to being able to metabolize pollutants, are used as an analytical technology to accelerate the decomposition of soils and contaminated sediments. This strain tested for their ability to utilize toluene at concentrations from 100 mg/L to 1200 mg/L and was capable of removing 100 mg toluene per liter in the liquid MSM by 47% in 24 h (Figure 1) and metabolizing toluene up to 59% in 48 h. By PCR amplification, a 1500-bp 16S rDNA gene fragment of strain ATHH39 obtained and submitted for sequencing. The blast search of this sequence indicated that ATHH39 matched at 99.9% to *Bacillus cereus* SATCC 14579 (T). Thus, ATHH39 was identified to be a *Bacillus cereus* sp.

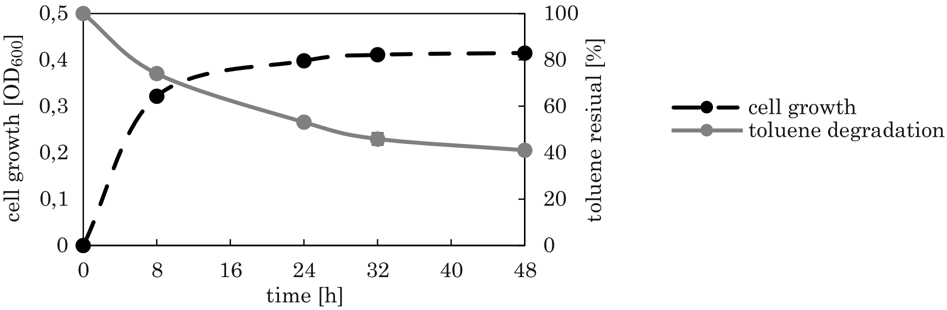

Fig. 1. Toluene-degrading and growth curves of strain ATHH39

RSM model development

Instead of optimizing medium composition by a one factor at a time approach, the statistical RSM design provides the opportunity to determine the optimal conditions, in any given parameters by establishing the relationship between factors and predicted responses. The RSM design was applied to obtain the precise factor values which results in the higher toluene degradation. The results are summarized in Table 2.

Table 2

RSM design for the three factors and their experimental results

Run order	Factors			Toluene degradation [%]	
	X_1	X_2	X_3	experimental	predicted
1	7	30	195.46	47.44	47.02
2	6	25	400	46.32	47.47
3	8	25	400	53.06	52.29
4	6	35	400	56.63	57.25
5	8	35	400	53.24	54.26
6	7	21.59	700	54.57	54.17
7	5.32	30	700	59.90	57.83
8	7	30	700	65.46	64.80
9	7	30	700	65.53	64.80
10	7	30	700	65.54	64.80
11	7	30	700	65.33	64.80
12	7	30	700	63.22	64.80
13	7	30	700	63.31	64.80
14	8.68	30	700	64.10	63.86
15	7	38.41	700	63.44	61.53

cont. Table 2

16	6	25	1000	54.77	55.37
17	8	25	1000	64.53	65.53
18	6	35	1000	59.75	62.15
19	8	35	1000	64.03	64.51
20	7	30	1204.54	64.16	62.27

Explanation: X_1 – pH; X_2 – temperature [^{o}C]; X_3 – toluene concentration (mg/L)

Toluene degradation

By applying multiple regression analysis on the experimentally determined data in Eq. (1), the regression coefficients were estimated and the following second-order polynomial equations was obtained using Design Expert software for optimum toluene degradation:

$$Y = 64.80 + 1.79X_1 + 2.19X_2 + 4.54X_3 - 1.40X_1^2 - 2.46X_2^2 - 3.59X_3^2 - 1.95X_1X_2 + 1.34X_1X_3 - 0.75X_2X_3 \quad (2)$$

The predicted optimum levels of X_1, X_2, and X_3 were obtained by applying regression analysis of Eq. (2), using Design Expert software, and they were 6.72 of pH, 33.16^{o}C of temperature and 824.15 mg/L of toluene concentration, respectively. The predicted value of toluene degradation was 65.85%. The coefficient of determination (R^2) value of the regression for the response related to significant effects on the model was 0.9601, which means that the sample variation of 96.01% of biomass production was attributable to the factors. The adequacy of the full quadratic model of toluene degradation also evaluated by ANOVA. Model summary statistics in Table 3 indicated the adequacy of the models including linear, 2-factor interactions, and quadratic terms. Linear and interaction models were significant at the 1% level.

Table 3

Analysis of variance for response surface quadratic model obtained from experimental design

Source	Sum of squares	DF	Mean square	*F*-Value	Prob. > *F*
Model	699.84	9	77.76	26.75	< 0.0001***
X_1	43.86	1	43.86	15.09	0.0030**
X_2	65.43	1	65.43	22.51	< 0.0008***
X_3	280.93	1	280.93	96.65	< 0.0001***
X_1^2	28.13	1	28.13	9.68	< 0.0110***

cont. Table 3

X_2^2	86.88	1	86.88	29.89	< 0.0003***
X_3^2	185.61	1	185.61	63.85	< 0.0001***
X_1X_2	30.48	1	30.48	10.49	< 0.0089***
X_1X_3	14.27	1	14.27	4.91	< 0.0511ns
X_2X_3	4.50	1	4.50	1.55	< 0.2419ns
Residual	29.07	10	2.91	–	–
Lack of fit	22.57	5	4.51	3.48	0.0989ns
Pure error	6.49	5	1.30	–	–
Cor total	728.90	19	–	–	–
Std. dev. = 1.70 Mean = 59.72 C.V.= 2.86 PRESS = 184.41		R-squared = 0.9601 Adj. R-squared = 0.9242 Pred. R-squared = 0.7470 Adeq. precision = 15.356			

Explanation: X_1 – pH; X_2 – temperature [°C]; X_3 – toluene concentration [mg/L] *values of "probability > *F*-Value" less than 0.05 indicates model terms are significant

SEM observations analysis

Free *Bacillus cereus* ATHH39 and Immobilized *Bacillus cereus* ATHH39 adhesion on the surface of MWCNTs in the presence of 100 mg/L toluene were observed using SEM (Figure 2). Figure 2(b) demonstrate that bacteria cells are trapped among the MWCNTs arrays bundles. It can be due to the interactions of bacteria cells with the external surfaces of MWCNTs arrays. Also, Figure 2 indicates that there are no major changes in the morphology of the bacteria cells after incubating with MWCNTs arrays. These SEM images reveal that MWCNTs clusters only capture the bacteria cells due to sieve mechanisms without any damage of the cell wall.

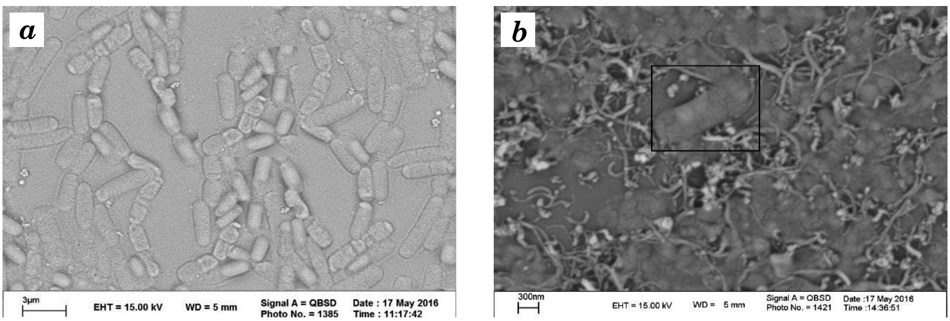

Fig. 2. Scanning electron microscopy image of (*a*) *Bacillus cereus* ATHH39, (*b*) immobilized *Bacillus cereus* ATHH39 by MWCNTs

No changes in the structures of the carbon nanotubes after bacterial immobilizing, which is the benefits of the method has been observed in other studies (KOLANGIKHAH et al. 2010). This observation differs from other studies (KANG et al. 2008a, KANG et al. 2008b). Using non-array CNTs have shown that CNTs rupture cell wall-membrane due to toxicity mechanisms such as oxidative stress (NEL et al. 2006) and physical damage (KANG et al. 2008a, KANG et al. 2008b) while this observation has not been observed here.

Effect of pH, temperature and toluene concentration

Cell immobilization has been used for biodegradation of several toxic chemicals. Immobilization facilitates the separation and retrieval of cells and leads to reuse and cost reductions (BAYOUMI 2009). MWCNTs used to immobilize *Bacillus cereus* strain ATHH39. Factors affecting toluene degradation of immobilized cells, such as pH, temperature and toluene concentration investigated. DÍAZ et al. (2002) reported that the immobilization of bacterial cells in comparison with free-living cells increased the rate of biodegradation of crude oil in a wider range of salinity. The pH optimum experiments for toluene degradation by free and immobilized cells performed at 30°C with toluene concentration of 700 mg/L and the results are shown in Figure 3. The optimal pH was 7 for free and immobilized cells, the degradation rates were 65.5% to 95.2% for free and immobilized cells, respectively.

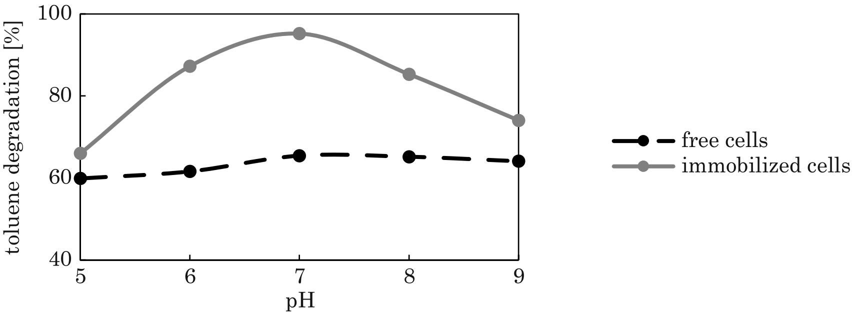

Fig. 3. Effect of pH on toluene degradation of free and immobilized cells

The immobilized cells have higher degradation rate constant and wider pH range than that of free cells. The changes of the toluene degradation of free cells were not as sharp as the immobilized cells. When pH was at about 7, toluene-degrading of free cells was completely inhibited. However, immobilized cells still maintained an acceptable degradation rate at the same pH. When pH increase or decrease the degradation rate of the immobilized and free cells were lower. To confirm that immobilized cells can also tolerate a wider temperature change, toluene degradation experiments performed at a temperature range from 20 to 40ºC at pH 7 with toluene concentration of 700 mg/L. Figure 4 shows the effect of temperature on toluene degradation of free and immobilized cells. The optimal temperature was (30ºC) and (35ºC) for free and immobilized cells, the degradation rates were 65.5% to 95.1% for free and immobilized cells, respectively.

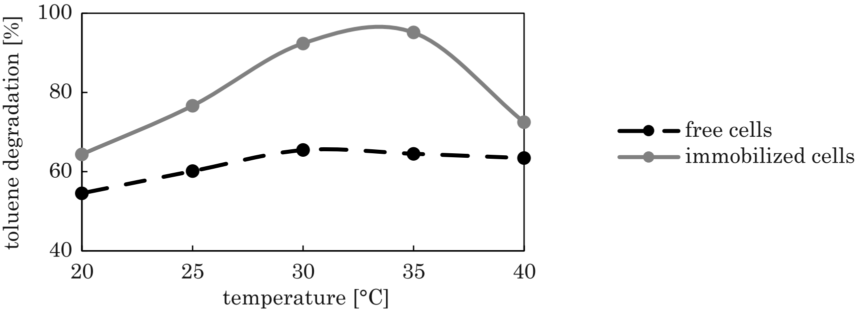

Fig. 4. Effect of temperature on degradation of free and immobilized cells

In the range of 30 to 40ºC, the immobilized cells showed a higher value of degradation rate than free cells. Especially when the temperature reached 35ºC, with the increase of the temperature, the curve of free cells declined slowly, but the curve of immobilized cells fell sharply. Figure 5 shows the effect of toluene concentration on toluene degradation of free and immobilized cells. The optimal toluene concentration was (700 mg/L) for free and immobilized cells, the degradation rates were 65.5% to 87.5% for free and immobilized cells, respectively. The immobilized cells have higher degradation rate constant. Especially when the toluene concentration reached 700 mg/L, with the increase of the toluene concentration, the curve of free cells and immobilized cells declined slowly.

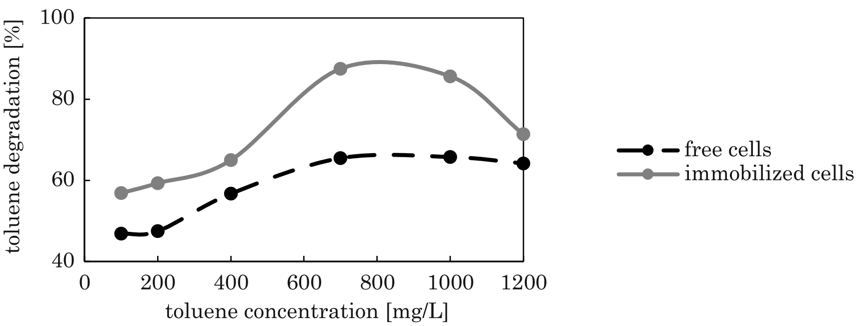

Fig. 5. Effect of initial toluene concentration on toluene degradation of free and immobilized cells

Conclusion

The results of this study showed the efficiency of 65.5% of toluene adsorption by free cells and the efficiency of 87.5% of toluene adsorption, by carbon nanotubes coated with *Bacillus cereus*. This is due to increase in biodegradation rate and active sites available for toluene adsorption through exopolysaccharides secreted from bacteria. The use of bacteria in the form of biofilm coating can play an effective and meaningful role in improving the absorption of toluene by carbon nanotubes.

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